Chloroform-treated filamentous phage as a bioreceptor for piezoelectric sensors

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Abstract

Affinity-selected filamentous bacteriophage was induced to spherical forms ("spheroids") by chloroform treatment and deposited to piezoelectric transducers by Langmuir-Blodgett to prepare biosensors for the detection of streptavidin and *S. typhimurium*. ELISA confirmed affinity-selected phage specificity for streptavidin. Spheroid induction was optimized to achieve greatest conversion yields as a function of solvent exposure time and concentration. Results from whole-virion agarose gel electrophoresis indicated 27-fold phage aqueous dilutions mixed with equal volumes of chloroform for 60 s at room temperature was adequate. Phage conversion to spheroids with subsequent binding to *S. typhimurium* was confirmed by transmission electron microscopy. Spheroids for streptavidin and *S. typhimurium* prepared as either pure monolayers of phage coat proteins or proteins reconstituted with phospholipids were evaluated by isotherm, elasticity, and transfer ratio analysis. Results showed that spheroids combined with phospholipids produced a phage coat monolayer possessing higher elasticity and transfer ratios than monolayers of phage coat proteins alone, resulting in spatially superior deposition to substrates and subsequent firm binding of *S. typhimurium* that followed mass theory for piezoelectric transducers. Scanning electron microscopy confirmed binding of streptavidin-coated beads and *S. typhimurium* to prepared biosensors. In summary, spheroid-based sensors could be an effective analytical method for detecting and monitoring quantitative changes of bacterial agents under any conditions that warrant their recognition.

1. Introduction

Filamentous bacteriophage affinity-selected for a ligand of choice from landscape libraries (Smith and Petrenko, 1997) are a demonstrated source of specific, selective probes that can be used in the detection of biological agents such as *Salmonella typhimurium* (Sorokulova et al., 2005) and as bioreceptors in conjunction with biosensor platforms (Olsen et al., 2005). The phage outer coat is predominately composed of approximately 4000 outer coat proteins (pVIII), which encase the viral ssDNA and comprise 87% of the phage mass (Bonnycastle et al., 2001). As prepared through phage display, the surface density of the phage binding peptides can be as high as 300 to 400 m^2/g and is comparable to the best-known absorbents and catalysts (Petrenko and Smith, 2000). Partial denaturation of filamentous phage with chloroform induces small, compact spherical forms (Manning et al., 1981) or "spheroids" mainly composed of the pVIII proteins, which retain their affinity for selected agents (Petrenko and Smith, 2000). Spheroids may be advantageous in situations that require multivalent, firmly attached, thinly layered bioreceptors such as probes for piezoelectric acoustic wave platforms. In this research, we have demonstrated the preparation of phage coat protein monolayers from spheroids and their subsequent deposition to piezoelectric substrates by Langmuir-Blodgett (LB) to prepare biosensors for the detection of the food safety threat agent *S. typhimurium*.

2. Materials and methods

2.1. Microorganisms

2.1.1. Bacteria

Salmonella typhimurium (ATCC 13311) was obtained from the American Type Culture Collection (Rockville, MD) and confirmed for identity, propagated, and maintained as previously described (Sorokulova et al., 2005).

2.1.2. Bacteriophage

Petrenko and Smith (2000) described affinity-selection of streptavidin-binding filamentous phage (clone 7b1 – displaying foreign peptide VPEGAFSS) from a landscape library. Specific, selective filamentous phage for S. typhimurium (clone E2 – displaying foreign peptide VTPPTQHQ) were culled from a landscape f8/8 phage library (Petrenko et al., 1996) through affinity-selection as described in Sorokulova et al. (2005). Wild-type vector f8-5, as described in Sorokulova et al. (2005), was utilized as a negative control.

2.2. Enzyme-linked immunosorbent assay (ELISA)

For direct ELISA, filamentous 7b1 (5.0×10^{11} virions/ml in TBS [50 mM Tris–HCl, 0.15 M NaCl, pH 7.5]) and f8-5 (5.0×10^{11} virions/ml in TBS) were adsorbed to alternating rows of 96-well polystyrene ELISA plates for 16 h at 4 °C. Plates were washed 5× with XX%TBS-Tween then filled with alkaline phosphatase-conjugated streptavidin (APSA) diluent (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20, 1 mg/ml BSA) containing APSA at graded concentrations. Following 45 min room

temperature incubation, plates were washed 10× with XX%TBS-Tween. Reaction was developed with 0.5 mg/ml p-nitrophenyl phosphate (NPP) then read every 3 min for 1 h (EL 808 microplate reader, BIO-TEK® Instruments) at OD_{405} and OD_{490} (Yu and Smith, 1996). The slope of color development was measured as a change in optical density (mOD/min).

For competitive ELISA, filamentous 7b1 and f8-5 were adsorbed to ELISA plates, washed, and assayed as described above with the exception that wells were filled with constant concentrations of APSA and graded concentrations of streptavidin, with the last well in each row containing a streptavidin concentration of zero as a control.

2.3. Spheroid preparation

Filamentous phage were converted to spheroids by chloroform denaturation for surface pressure–area (Π–A) isotherms and deposition to piezoelectric quartz crystal microbalances (QCM) by LB. Spheroids were prepared <1 h prior to isotherm measurement and/or biosensor preparation. All materials were autoclave sterilized prior to use. Procedures were performed in a biological safety cabinet with aseptic technique.

2.3.1. Filamentous phage conversion to spheroids

The partial-denaturation method of Manning et al. (1981) with modifications was used to convert filamentous phage 7b1 (1.0×10^{13} virions/ml in TBS) and E2 (2.68×10^{12} virions/ml in PBS [0.15 M NaCl, 5 mM NaH_2PO_4, pH 7.0]) to spheroids. Diluted phage were combined with equal volumes of spectrophotometric-grade $CHCl_3$ (99.8% A.C.S.,

Sigma–Aldrich, Milwaukee, WI) then induced to spheroids by gentle vortexing 5× – 6×

(5 s intervals) for 1 min. All reactants were at room temperature. The top phase

(aqueous) containing spheroids, as well as the phase interface, were retained for analysis

by electrophoresis and the preparation of LB monolayers for isotherms and biosensors.

2.3.2. Spheroid electrophoresis

Conversion of filamentous phage to spheroids was monitored by whole-virion

agarose gel electrophoresis (AGE). Ten microliter aliquots of spheroids and 4 μl aliquots

of stock filamentous phage 7b1 (1.0×10^{14} virions/ml in TBS) and E2 (6.7×10^{13}

virions/ml in PBS), both containing 20% (v/v) bromophenol blue dye, were resolved in

0.8% (w/v) agarose gels (DNA high-melting agarose prepared with 0.05 M NaH_2PO_4 and

1mM $MgCl_2$, pH 7.5; Fisher Scientific, Fair Lawn, NJ) using a Mini–sub® cell GT

system (Bio–Rad Laboratories, Hercules, Ca) with 0.05 M NaH_2PO_4 buffer containing

1mM $MgCl_2$, (pH 7.5). Compounds used to prepare buffer and gels were analytical grade

or higher. Spheroids were prepared <1 h prior to use. λ_{BstEII} DNA heated at 70 °C for 10

min was used as a size standard. AGE was performed for 1.5 h at 50 V. Gels were

rinsed in double-deionized water (DDH_2O) then treated by immersion in 0.2 N NaOH for

1 h, 1 M Tris-HCl (pH 7.5) for 15 min, and 0.05 M NaH_2PO_4 buffer with 1mM $MgCl_2$,

(pH 7.5) containing 7 μl nucleic acid gel stain (SYBR® Green I, 10000X concentration

in DMSO, Cambrex Bio Science, Rockland, MD) for 1 h while rocking. Following final

rinse, filamentous phage and spheroid DNA were visualized (521 nm) using a KODAK

290 Electrophoresis Documentation and Analysis system (Eastman Kodak Company,

Rochester, NY), equipped with a DR40 Kodak camera, DR40 amber camera filter (Clare

Chemical Research, Denver, CO) and dark reader transilluminator (Clare Chemical Research). Images (exposure = 8 s) were captured and processed electronically using Kodak 1D software (Ver. 3.5.3, Eastman Kodak Company).

2.3.2.1. Timed chloroform exposure studies. AGE was utilized to determine the optimal exposure period of filamentous phage to $CHCl_3$ for conversion to spheroids. E2 filamentous phage (6.7×10^{13} virions/ml in PBS) was diluted to concentrations of 2.2×10^{13} virions/ml and 1.1×10^{13} virions/ml with PBS then induced to spheroids with $CHCl_3$ using either a 1 min or 3 min exposure duration.

2.3.2.2. Phage-CHCl₃ concentration studies. AGE was also used to qualify the degree to which filamentous phage contract into spheroids when titrated against constant amounts of $CHCl_3$. E2 phage (6.7×10^{13} virions/ml in PBS) was diluted to five separate logarithmic concentrations ranging from $2.76 \times 10^{11} - 2.2 \times 10^{13}$ virions/ml then induced to spheroids using $CHCl_3$.

2.3.3. Spheroids reconstituted with phospholipids

Following initial isotherm analysis of 7b1 and E2 spheroids as pure protein monolayers, E2 spheroids were also reconstituted with 1,2–diphytanoyl–sn–glycero–3–phosphocholine (PC) (Avanti Polar Lipids, Inc., Alabaster, AL) as an alternate monolayer preparation. Six hundred microliters of prepared E2 spheroids in aqueous solution were combined with an equal volume of PC previously reconstituted in hexane (1 mg/ml). The mixture was vortexed vigorously to homogenous emulsion. Following standing

formation of an interface, the top phase (aqueous) containing spheroids and PC was retained for preparation of monolayers for isotherms and biosensors.

2.4. Isotherm and biosensor preparations

The KSV 2200 Langmuir surface film balance and general procedures used to prepare Π–A isotherms and monolayers for deposition to quartz substrates has been thoroughly described (Olsen, 2000; Olsen et al., 2003; Pathirana et al., 2000).

2.4.1. Spheroid Isotherms

Spheroids in solution, prepared alone (7b1 and E2) or reconstituted with PC (E2 only), were brought to room temperature if applicable, vortexed to a homogenous mixture, then pipetted to the subphase surface ($\approx$ 10 μl drops) via a cleaned, wetted glass rod until a surface pressure of 0.5 – 0.9 mN/m was obtained. The amount of spheroid solution pipetted to the surface was variable but generally between 100 – 200 μl; pure spheroids required greater volumes and those reconstituted with PC required less. A constant temperature circulator maintained the subphase at 19 ± 0.1 °C. Following 10 – 20 min for equilibration and evaporation of hexane (E2 spheroids - PC), the surface layer was compressed at 30 mm/min.

Data files from the processing unit of the film balance instrument were analyzed off-line using Excel 2000 (Microsoft, Seattle, WA) and Origin® 6.0 (Microcal Software, Northampton, MA). ASCII file data for each isotherm, including compression barrier position (mm), surface pressure (mN/m), and trough area (mm^2) was imported into an

individual spreadsheet. The area (%) of the trough, and compressibility modulus, K, (mN/m) for the elasticity plot, were derived from this data. The isotherm was plotted as a line graph depicting surface pressure, Π, as a function of trough area, A. Elasticity was plotted as a line graph depicting compression modulus, K, as a function of the surface pressure, Π.

2.4.2. Phage coat biosensors

Deposition of spheroids onto resonators as either pure protein monolayers or in combination with PC followed the procedure described by Olsen (2000), Olsen et al. (2003) and Pathirana et al. (2000) for *Salmonella* polyvalent-O antibodies with the following exceptions. An initial surface pressure of 8 – 10 mN/m was prepared using 600 – 2000 µl of spheroid solution, and a compressed surface pressure of 36 mN/m (E2), 38 mN/m (E2 -PC), or 30 mN/m (7b1) was used for deposition as determined by isotherm/elasticity analysis. Approximately 3 – 23 monolayers were deposited onto resonators; variability in the number of monolayers depended upon the efficiency of spheroid transfer from the subphase to the resonators as determined by transfer ratio (TR).

2.5. Biosensor binding measurements

The Maxtek acoustic wave device (AWD), accompanying QCM piezoelectric resonators, binding measurement procedures, and data analysis have been thoroughly described (Olsen, 2000; Olsen et al., 2003; Olsen et al., 2005; Pathirana, et al., 2000).

Biosensors prepared with 7b1 phage were tested with logarithmic concentrations of ≈ 1μm streptavidin– or bovine serum albumin (BSA)–coated polystyrene beads (Bangs Labs, Inc., Fishers, IN). Biosensors prepared with E2 phage were evaluated with bacterial suspensions prepared from an overnight culture of *S. typhimurium* as described (Olsen et al., 2005). Monitors were calibrated prior to use. All experiments were conducted at room temperature.

2.6. Microscopic analyses

Transmission Electron Microscopy (TEM) allowed visual characterization of E2 spheroids both as independent entities and in complex with *S. typhimurium*. Prepared spheroids independent of *S. typhimurium* were diluted 1:100 in DDH_2O. Spheroids in complex with bacteria were prepared by incubating 1 ml of spheroids with *S. typhimurium* (10^8 cells) for 10 min while rotating then washing 10× with PBS and diluting 1:100 in DDH_2O. Ten microliter aliquots of the preparations were incubated with formvar/carbon-coated grids (EMS, Hatfield, PA) for 45 s – 1 min; excess fluid was removed by absorption to filter paper. Grids preps were washed and negatively stained with either filter-purified (0.22 μm) 2% (w/v) uranyl acetate (pH ≈ 4.5) or 2% (w/v) phosphotungstic acid (pH 7.0) for 3 – 5 min, then examined under a Philips EM 301 TEM (Netherlands) at 60 kV. Hand-developed micrographs were prepared to digital format by scanning and processing with Adobe® Photoshop® (Ver. 5.0LE, Adobe Systems).

Assayed biosensors were subjected to Scanning Electron Microscopy (SEM) and prepared as described (Olsen et al., 2005) except that sensors were exposed to 2% osmium tetraoxide for 1 h prior to air-drying and sputter coating.

3. Results and discussion

3.1. Phage specificity confirmation

Streptavidin is a slightly acidic tetrameric protein produced by *Streptomyces avidinii*, and binds biotin with high affinity. It was utilized in this work as a model threat agent for proof of concept preparation and testing of spheroids as probes. Spheroids for streptavidin were prepared from an Ff class filamentous phage (designated phage clone 7b1) previously affinity-selected for streptavidin from a landscape phage library (Petrenko and Smith, 2000). Phage 7b1 displays the foreign peptide VPEGAFSS at the N-terminus of all 4,000 pVIII outer coat proteins.

Direct ELISA was used to confirm the specificity and dose-dependent binding of 7b1 to streptavidin in comparison to wild-type phage f8-5. 7b1 and f8-5 phage were adsorbed to separate ELISA wells and streptavidin was added in the form of APSA. As shown in Fig. 1A, binding between 7b1 and streptavidin was strong and dose-dependent ($R^2 = 0.997$) while f8-5 demonstrated no significant signal. Competitive inhibition ELISA between APSA and streptavidin for 7b1 verified that phage-APSA interaction was due solely to the streptavidin component (Fig. 1B). These findings confirm those of Petrenko and Smith (2000), whose ELISA results also demonstrated specific binding of a

synthetic peptide corresponding to the N-terminal portion of a streptavidin-binding phage.

The affinity selection, specificity, and selectivity of phage clone E2 for *S. typhimurium* has been demonstrated and discussed previously (Sorokulova et al., 2005).

3.2. Spheroid preparation

Previously, filamentous phage was readily adsorbed to gold QCM electrodes as a bioreceptor for the preparation of *S. typhimurium* sensors (Olsen et al., 2005). While the majority of these sensors elicited frequency responses indicative of mass loading when challenged, some suffered from a flat or inverse responses thought to result from poor spatial orientation of phage on the sensor as a result of an uncontrolled deposition process. To alleviate this problem, vesicle-like spherical forms of filamentous phage, termed here as "spheroids," were prepared by chloroform partial denaturation using the method of Manning et al. (1981) for preparation and deposition of monolayers to QCM by LB. LB is a controllable film deposition technique that can be used for the production of spatially organized monolayers for sensors (Bailey et al., 2002; Naylor 2002; Petrenko and Vodyanoy, 2003; Samoylov et al., 2002a, 2002b) and is a method we have utilized successfully in the past to prepare functional *S. typhimurium* QCM sensors using *Salmonella* polyvalent-O antibodies (Olsen, 2000; Olsen, et al., 2003; Pathirana et al., 2000). Although filamentous phage doesn't contain a lipid component, $CHCl_3$ can act on hydrophobic interactions between its major coat protein subunits (pVIII), causing it to contract from an infectious filament into a non-infective, hollow sphere. The

transformation results in a drastically altered surface architecture whereby the α-helix content of pVIII decreases from 90% to 50-60% (Griffith et al., 1981; Roberts and Dunker, 1993) while two-thirds of the DNA is extruded (Griffith et al., 1981; Manning et al., 1981). However, the binding efficiency of partially denatured phage has been shown to be comparable to that of the native filamentous phage (Petrenko and Smith, 2000).

Conversion from filaments to spheroids by chloroform denaturation was monitored by whole-virion AGE for both 7b1 and E2 (Fig 2A). Initially, non-treated (lanes 2, 7) and $CHCl_3$-treated (Lanes 3, 6) 7b1 spheroids were electrophoresed at low current then either subjected to alkaline (NaOH) treatment (Lanes 1-3) or left untreated (Lanes 6-8) prior to DNA staining. Like $CHCl_3$, the NaOH treatment causes partial-denaturation of the coat proteins, exposing the DNA from the native phage (Lane 2); however, it does not induce spherical forms. Non-treated ($CHCl_3$ or NaOH) filamentous phage DNA is protected from staining by intact major coat proteins (Lane 7). Bands corresponding to spheroids produced by chloroform denaturation are visible regardless of alkaline treatment (Lanes 3, 6 – A and B) and because of their shape, migrate further than filaments. The morphology of the streptavidin spheroids was confirmed by TEM (Fig. 3A).

Further studies with E2 spheroids revealed that the highest preparation yield occurs when there is a concurrent decrease in exposure duration and concentration of phage in relation to chloroform (Fig. 2B - lane 3). A one-minute exposure appears to be sufficient for phage conversion, in good agreement with Manning et al. (1981) and previous observations by Petrenko and Smith (2000) and Petrenko et al. (1996). Spheroids were easily prepared at room temperature; however, in contrast to the findings

of Manning et al. (1981), intermediate forms (I-forms) were also readily apparent (Fig. 3B). DNA extrusion was also confirmed by TEM (Fig. 3C). TEM was also used to demonstrate binding of E2 spheroids to *S. typhimurium* in solution (Fig. 3D). To further investigate yield as a function of phage concentration, logarithmic dilutions of stock phage were prepared and subjected to equal volumes of chloroform as before, then again analyzed by whole-virion AGE. Densitometry revealed that 27× – 81× dilution of phage (Fig. 2C - lanes 5, 6) results in the highest yields of spheroids. Spheroids are not prevalent in the aqueous phase of highly concentrated phage preparations (Fig. 2B - lanes 1 and 4; Fig. 2C – lane 2). Rather, they are found in the phase interface (Fig. 2C - lane 7, band B and corresponding Fig. 3E) interlocked with large fibrils of filamentous phage that do not convert to spheroids. Speculating, increased numbers of spheroids using decreased concentrations of phage may be attributed to phage-solvent interaction opportunity. Phage aggregation in solution (Sorokulova et al., 2005) appears to decrease with aqueous dilution, resulting in greater numbers of free filaments in solution that may have greater opportunity to interact with solvent, resulting in correspondingly increased yields of spheroids in the aqueous phase. A second band, A, noted in Figs. 2B and 2C corresponds exactly with the migratory face of filamentous phage not subjected to $CHCl_3$ denaturation (Fig. 2C – lane 1). This band would appear to be I-forms seen by TEM (Figs 3B, 3C). Fig. 2C – lane 1 confirms filamentous phage aggregation in solution as a large, prevalent band that barely migrates out of the well.

3.3. Isotherm analysis

Based on AGE results, a 27-fold phage dilution mixed with an equal volume of chloroform for 1 min was used to prepare spheroids for LB isotherm analysis and biosensor. When added to the subphase solution using a glass rod and pipette, it is thought that the spheroids rupture at the air-liquid interface from surface tension, much like lipid vesicles, to form a monolayer of pVIII phage coat proteins or "skinned phage."

Details of pVIII conformation at both the air-liquid of the LB subphase and solid-liquid interfaces of the sensor can be deduced from the amino acid sequence of 7b1 pVIII:

N–A<u>VPEGAFSS</u>DPAKAAFDSLQASATE<u>YIGYAWAMVVVIVGATIGIKLFKKFTSKAS</u>–C.
  1   2

The octapeptide insert, VPEGAFSS (underlined region 1), is located between residues 1 and 10 at the N-terminal portion of the protein. As shown in Fig. 4, Garnier-Robson and Chou-Fasman calculations indicate three α-helical regions (underlined region 2) and a central hydrophobic region. This hydrophobic region spans the host bacterial cell membrane during phage assembly. The terminal ends of the protein are somewhat hydrophilic and most of the amphipathic and flexible regions of the peptide correspond with the peptide's ends. The antigenic index confirms the most probable antigen-binding region lies on the N-terminus where the octapeptide insert is located.

Fig. 5 depicts a hypothetical arrangement of skinned phage at the air-liquid interface. The hydrophilic terminal α-helices interact with the subphase while the central hydrophobic region remains above the interface. Phage coat proteins deposited to the gold electrode surface of piezoelectric resonators may be arranged in a similar

conformation to their native structure in the intact phage filament, where the positively charged C-terminal lysine residues interact with the negatively charged gold surface (Fig. 6), resulting in exposure of the pVIII peptide insert to solvent.

Isotherms of monolayers prepared from spheroids were measured by gradually compressing a prepared surface film of skinned phage at constant rate and temperature into a defined area (LB trough) while monitoring changes in surface pressure. The Π is a two-dimensional analogue of repulsive pressure exerted by the phage coat proteins, as described by the equation,

$$\Pi = \gamma - \gamma_0 \tag{1}$$

where, γ is the surface tension in the absence of a surface film, and γ_0 is the surface tension of the subphase in the presence of a surface film. Eq. (1) is plotted as a function of the subphase surface area available to each particle (Fig 7A). In comparison to the standard, arachidic acid (AA), 7b1 spheroids (7b1) yielded a biphasic curve, having a very long gaseous phase to around 20 mN/m followed by a steep liquid-condensed region. Ultimately, a solid-phase was reached $\approx$ 50mN/m – extremely high for pure protein monolayers, indicating a very stable system (Davies and Rideal, 1963). E2 spheroids (E2) were also characterized by a very long gaseous phase, requiring over 60% compression before a liquid-expanded state was reached at 10 mN/m. A liquid-condensed state was visible between 29 and 35 mN/m and a solid phase was reached at 35 mN/m. This solid phase Π is also high for a pure protein monolayer and greater than that of *Salmonella* antibodies (SAB) used previously to prepare biosensors for the

detection of *S. typhimurium* (Olsen, 2000). Π-A isotherms of monolayers prepared from E2 spheroids with PC (E2-PC) revealed a shorter gaseous phase in comparison to pure spheroids, probably due to earlier film organization as a result of the incorporated phospholipids. A liquid-expanded state was again reached at 10 mN/m. A liquid-condensed state was visible between 29 and 40 mN/m and a solid phase was seen between 40 and 46 mN/m.

Π–*A* isotherm data was further utilized to determine the compressibility modulus, *K*, of the film, which is a quantitative elastic property of the spheroids describing its compactness and stability and is as a function of Π,

$$\text{Elasticity, } K = -A\left(\frac{d\Pi}{dA}\right)_T \tag{2}$$

where, A = area of the film = $(450 - 1) \times 150$ [mm^2]; Π = surface pressure, and T = temperature at compression. Elasticity provides a more lucid characterization of the best Π at which to compress the monolayer for optimal packing and transfer to the piezoelectric substrates, especially for monolayers prepared from spheroid- or antibody-lipid mixtures, where it is becomes necessary to consider the immobilization of protein in the lipid-based film and its two dimensional compressibility. When Eq. (2) is plotted as a function of Eq. (1) (Fig. 7B), an optimal Π from the curve of maximum K (K_{max}) is derived (Table 1). For 7b1 spheroids (7b1), there were two maxima, the largest of which reached ≈ 46.9 mN/m, equating to an optimal Π of 41.22 mN/m. For monolayers prepared from E2 spheroids (E2), optimal Π was 35.98 mN/m, corresponding to the

highest Π obtained in the solid phase on the isotherm. This was three times higher than

optimal Π for *Salmonella* antibodies (SAB) (11.23 mN/m). E2 PC spheroids (E2-PC)

were very comparable to those of 7b1, possessing optimal Π of 37.81 mN/m. This was

slightly higher than that of spheroids alone and again, is a reflection of the added

phospholipids. Based on isotherm and elasticity analysis, a compressed surface pressure

of 36 mN/m (E2), 38 mN/m (E2-PC), or 30 mN/m (7b1) was used to deposit phage coat

proteins to QCM for the preparation of biosensors.

3.4. Biosensor preparation and testing

3.4.1. Monolayers transfer efficiency

Typical cumulative transfer efficiencies of phage coat monolayers to QCM are

shown as a function of the transfer ratio in Table 2, in comparison to the standard

arachidic acid. Arachidic acid, exhibited a cumulative TR of 6.7 for 7 (96%) deposited

layers, equating to near-perfect assembly of monolayers. In contrast, 7b1 and E2 phage

coat proteins possessed much lower transfer efficiencies (<32%), indicating that non-

ideal monolayers were transferred. This was not surprising since all pure spheroid

preparations required 10 times the amount of material compared to arachidic acid to

achieve a sufficient initial surface pressure. Pure phage coat proteins probably possess

poor buoyancy, sinking into the subphase during the compression and deposition process.

However, in comparison to *Salmonella* antibodies (10%), monolayers prepared from

spheroids more readily deposited to QCM. E2 phage coats proteins combined with PC

had the highest transfer efficiency (40%), indicative of the incorporated phospholipids that lend greater buoyancy to the skinned phage.

3.4.2. Biosensor analysis

Fig. 8A shows typical 7b1 phage coat sensor responses to increasing concentrations of streptavidin-coated beads. For each concentration, the sensor quickly comes to steady-state equilibrium within several hundred seconds following specific binding. Correlation analysis of mean steady-state voltage readings plotted as a function of bead concentration (Fig. 8B) reveals a linear dose-response relationship ($r = 0.997$, p <0.05) from $2.1 \times 10^4 - 2.1 \times 10^8$ beads/ml. Biosensor sensitivity, measured as the slope of linear regression, was vastly greater for streptavidin-coated beads (48.3 mV) than BSA-coated beads (5.1 mV). SEM confirmed streptavidin-coated bead attachment to assayed sensors (Fig. 10A). Based on these results, sensor production and testing was initiated using spheroids prepared from filamentous phage E2. E2 phage coat sensor dose-responses to *S. typhimurium* were comparable to those of streptavidin-coated beads (responses not shown) for variable cell concentrations over a range of $10^1 - 10^7$ cells/ml. Fig. 9A depicts experimental correlation coefficient, r, of 15 assayed E2 sensors as a function of sensitivity. The correlation coefficient ranged from + 0.99 (direct linear correlation) through 0 (no linear correlation) to − 0.99 (inverse linear correlation) for positive, zero, and negative sensitivities, respectively. Only a minority of sensors (20%) possessed goodness of fit and sensitivity greater than established background ($r \geq 0.90$, sensitivity ≥ 2.5 mV/decade − data not shown). Further studies with sensors produced from E2 spheroids reconstituted with phospholipids were more favorable. Fig. 9B

depicts the experimental correlation coefficient, r, of 24 assayed E2-PC sensors as a

function of sensitivity. The majority of these sensors (62.5%) possessed goodness of fit

and sensitivity greater than established background ($r \geq 0.90$, sensitivity ≥ 2.5 mV/decade

– data not shown). Importantly, there is a lack of negative linear sensor responses as seen

for E2 phage coat sensors prepared without phospholipids. This indicates that

phospholipid incorporation produces superior phage coat deposition and spatial

orientation as a result of increased monolayer rigidity and transfer efficiency, thus

allowing firm binding of *S. typhimurium* (Fig. 10B) in comparison to a control (Fig. 10C)

that follows mass theory for QCM-based sensors.

4. Conclusions

Overall, this work demonstrates the feasibility of preparing biosensors for the

detection of streptavidin and *S. typhimurium* based on affinity-selected bacteriophage

treated with chloroform as a bioreceptor for QCM-based sensors. We have provided the

first report on LB phage coat monolayers and the application of "phage-skinning" to the

production of biosensors. With further refinement, these sensors could be an effective

analytical platform for detecting and monitoring quantitative changes of bacterial agents,

including food and bioterrorism applications, since the nature of the bioreceptor layer

holds potential development against any bacteria, virus or toxin to which a corresponding

phage could be affinity-selected for.

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Table 1

Optimal surface pressure corresponding to maximal compressibility modulus of

biopolymers at 20 °C.

Biopolymer	Π (mN/m)	K_{max} (mN/m)
Salmonella Ab[a]	11.23	29.88
Spheroids – 7b1	41.22	47.51
Spheroids – E2	35.98	20.57
Spheroids – E2-PC[b]	37.81	48.64
Arachidic acid[c]	59.93	351.60

[a]*Salmonella* somatic-O polyvalent antibodies, Denka Seiken Co., LTD., Tokyo, Japan.

[b]E2 spheroids prepared by chloroform denaturation and reconstituted in phosphocholine

(PC).

[c]pH corrected to 10.0.

Table 2

Cumulative deposition of biopolymers onto quartz resonators (QCM) as a function of transfer ratio (TR) at 20 °C.

Biopolymer	Cum. TR	Layers deposited	Efficiency, (%)[a]
Salmonella Ab[b]	0.9	9	10
Spheroids – 7b1	1.6	5	32
Spheroids – E2	7.2	23	31
Spheroids – E2 PC[c]	4.4	11	40
Arachidic acid	6.7	7	96

[a]Represents typical deposition data for a single batch (n = 4) of sensors; some batches had better or worse efficiencies.

[b]*Salmonella* somatic-O polyvalent antibodies, Denka Seiken Co., LTD., Tokyo, Japan.

[c]E2 Spheroids prepared by chloroform denaturation and reconstituted in phosphocholine (PC)

Fig. 1A

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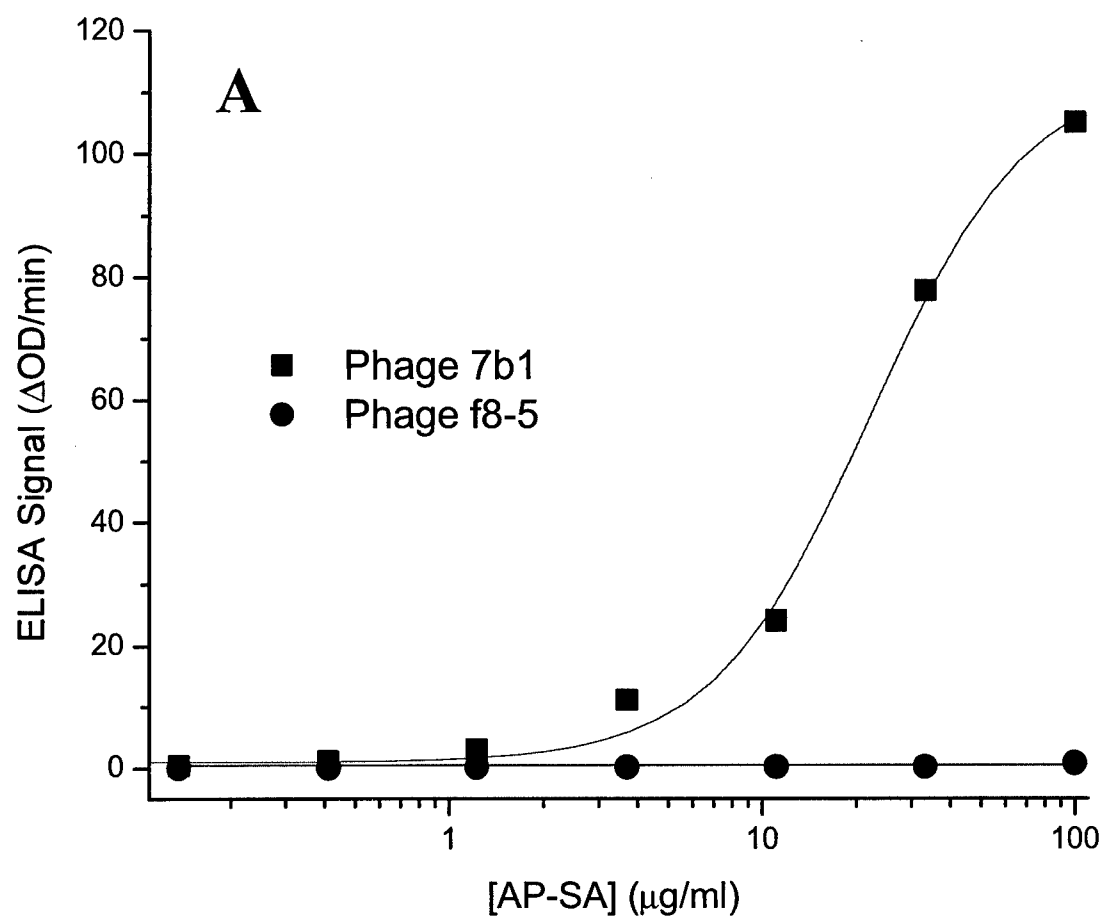
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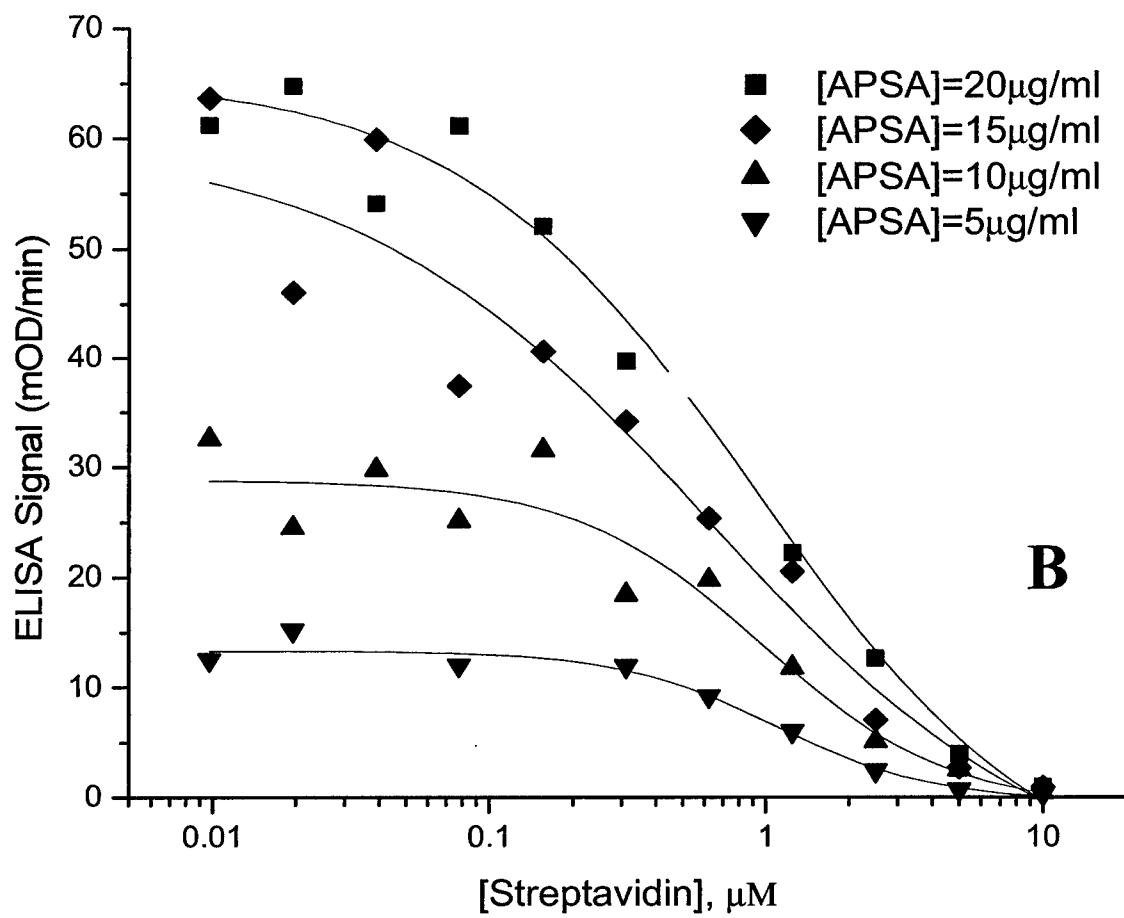
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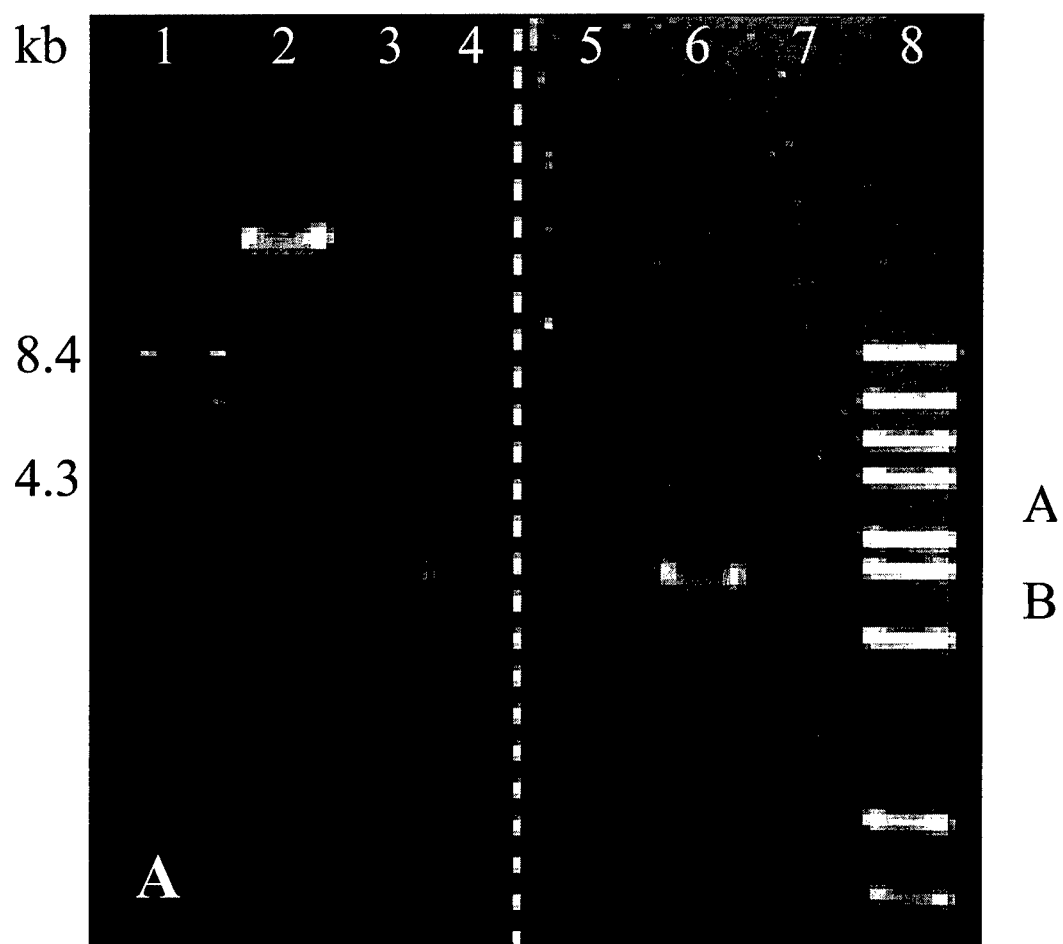
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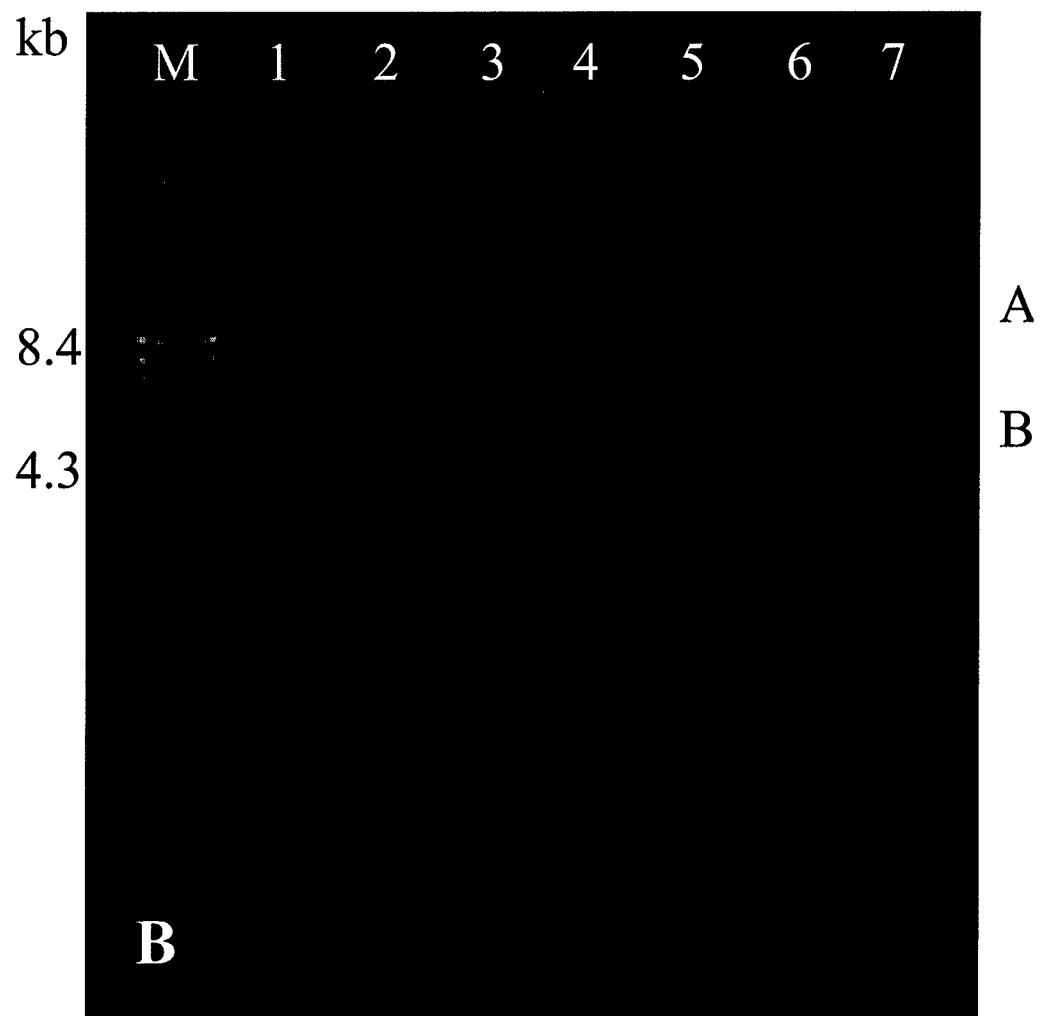
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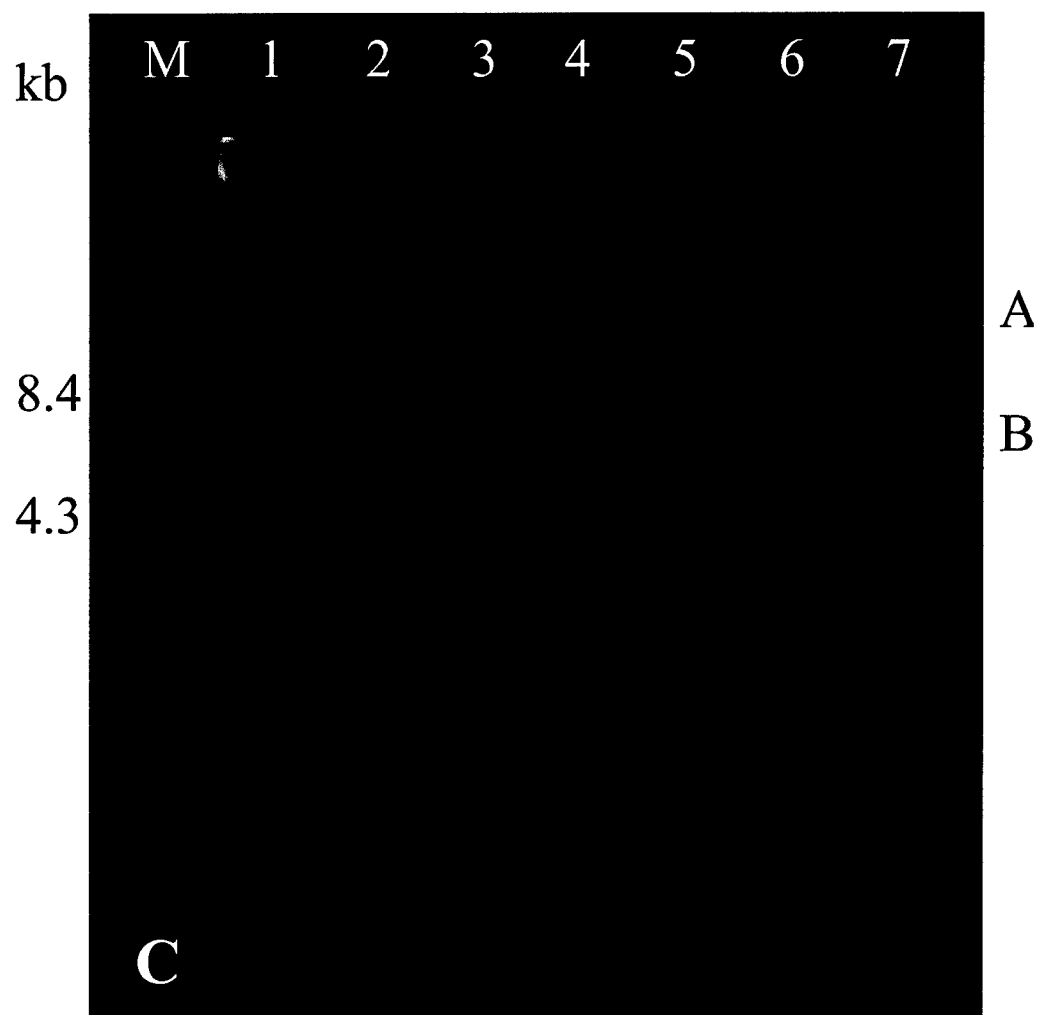
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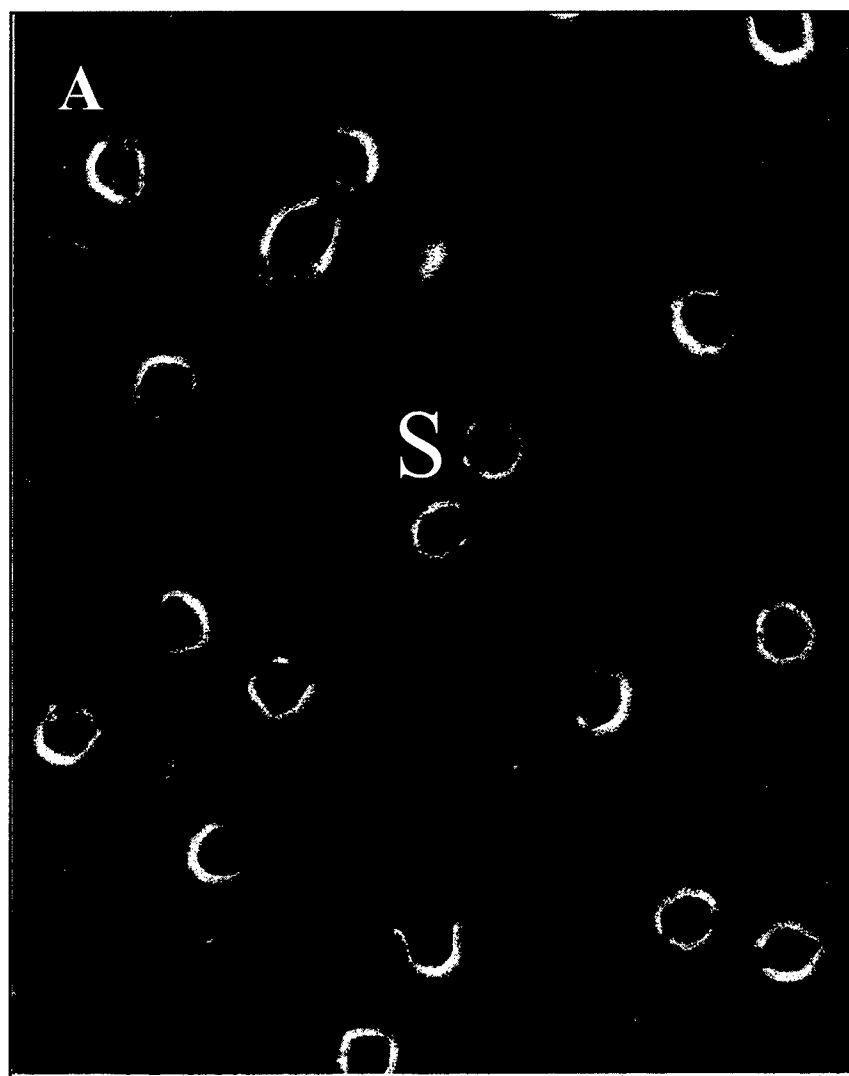
Fig. 3B

Fig. 3C

Fig. 3D

Fig. 3E

Fig. 4

Amino Acid # (beginning at N-terminus)
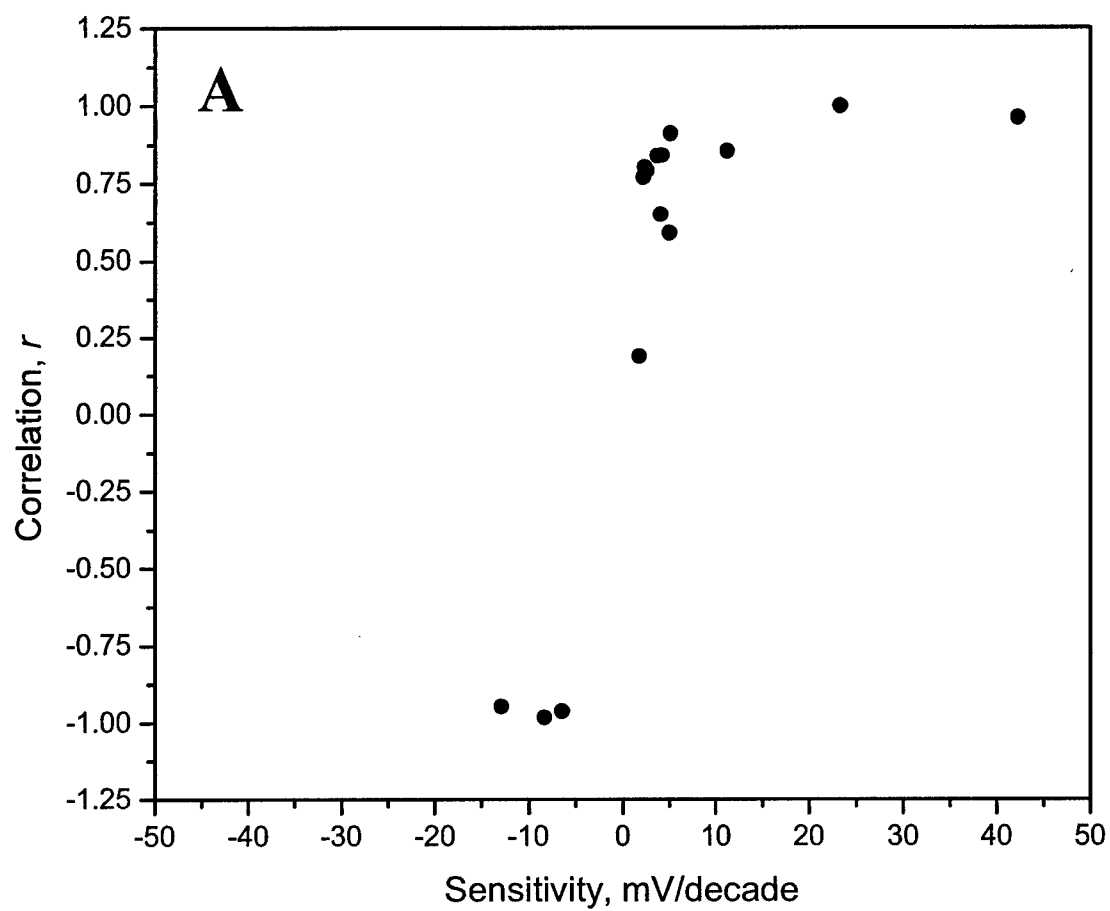
Fig. 5

AIR

Hydrophobic
domain

WATER
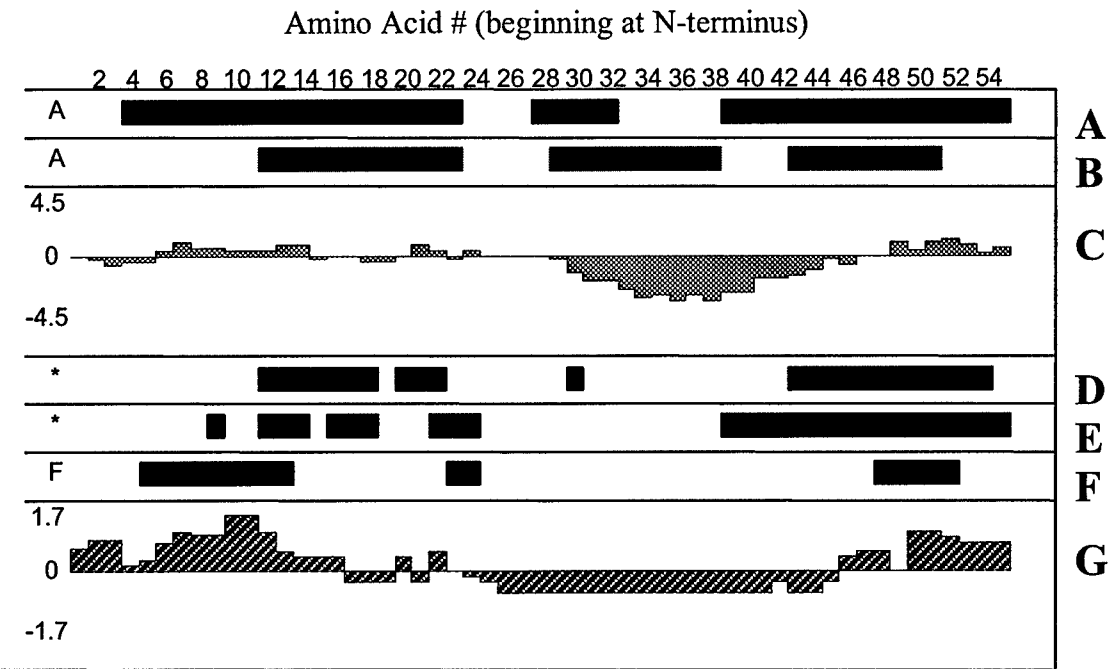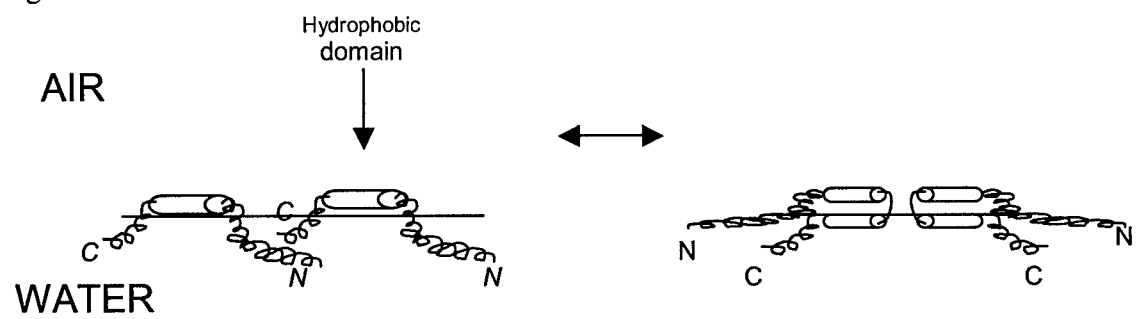
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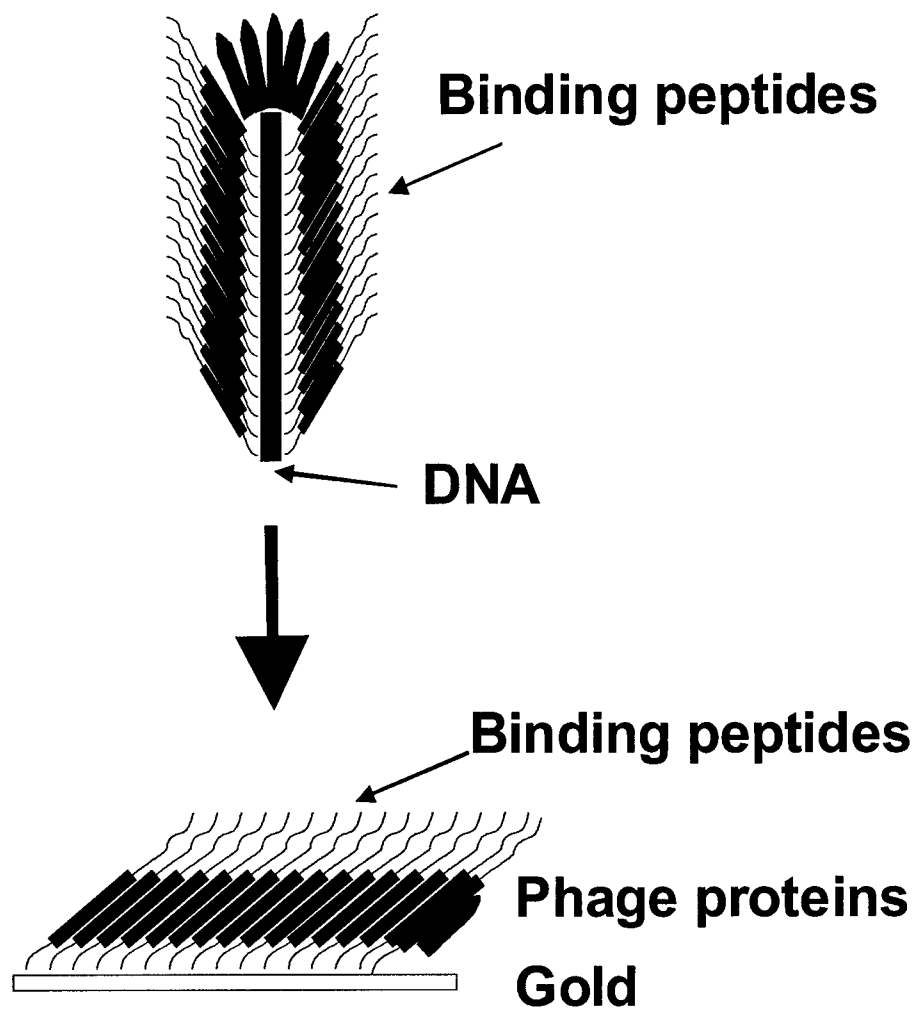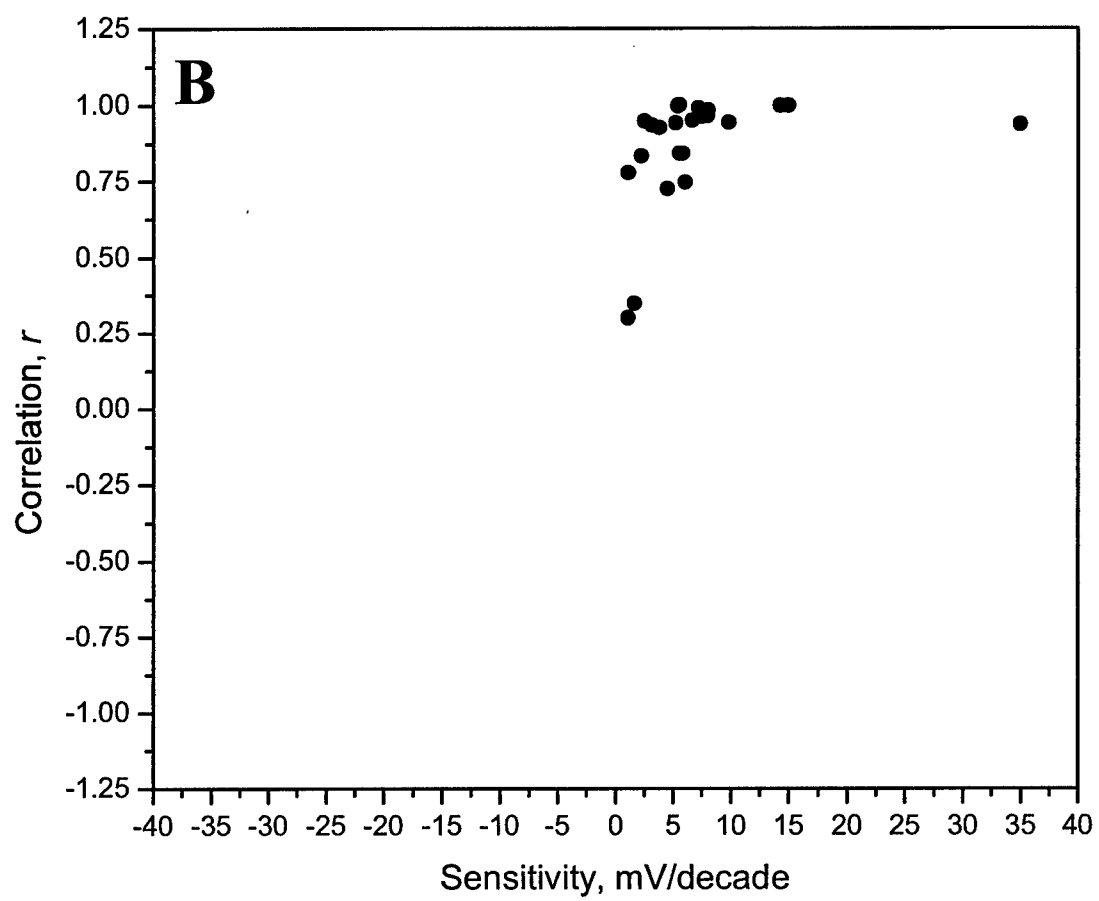
Fig. 7A
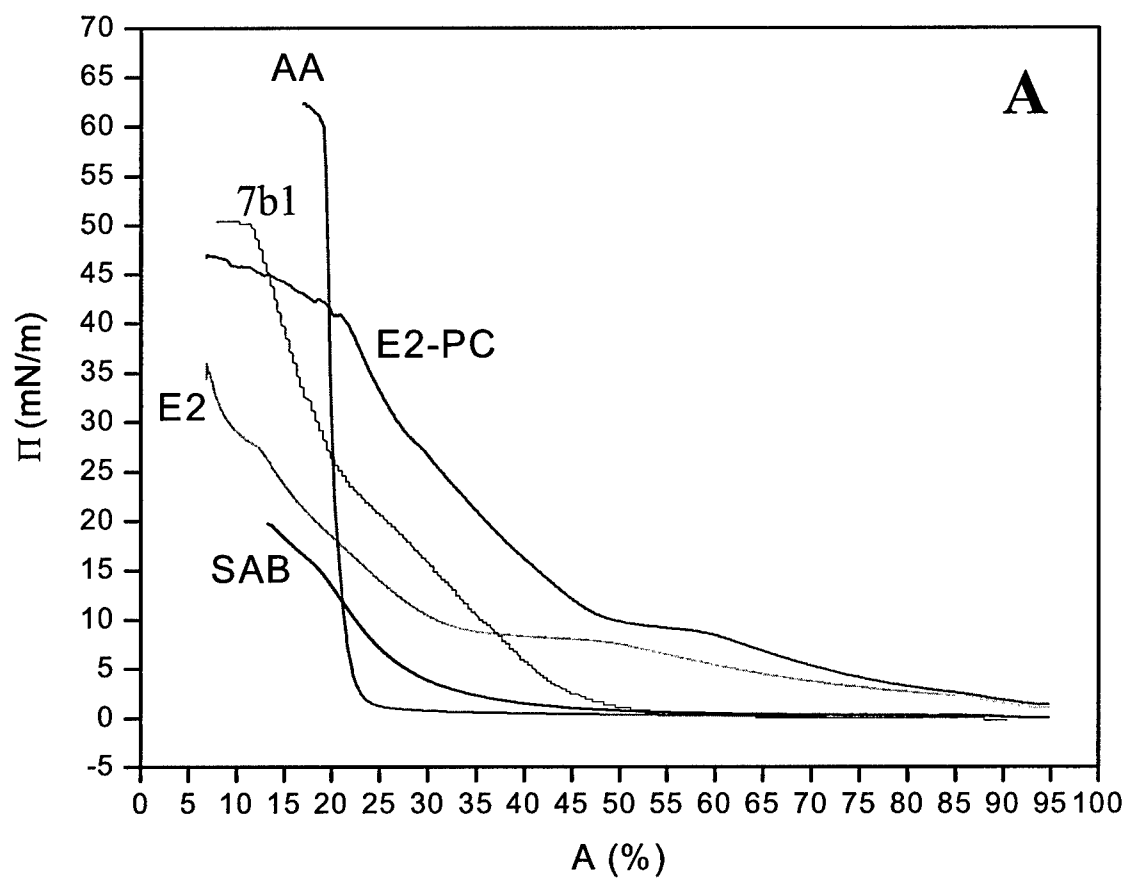
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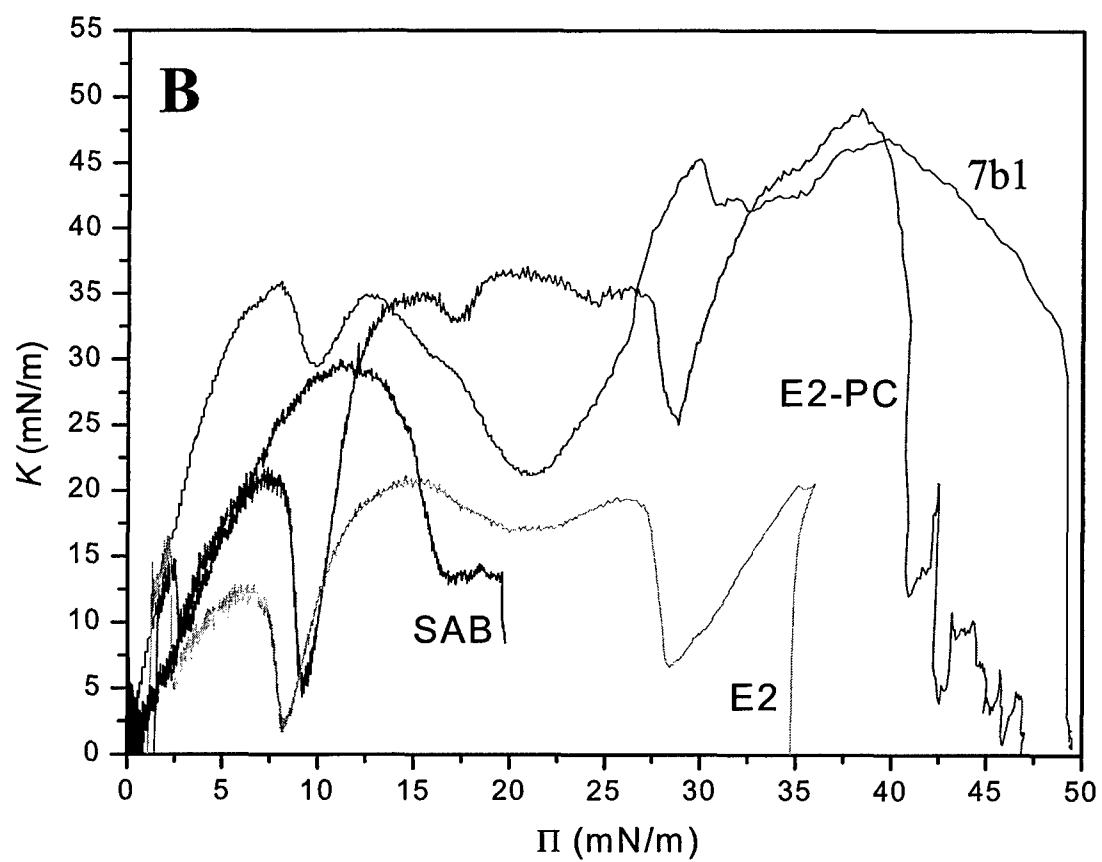
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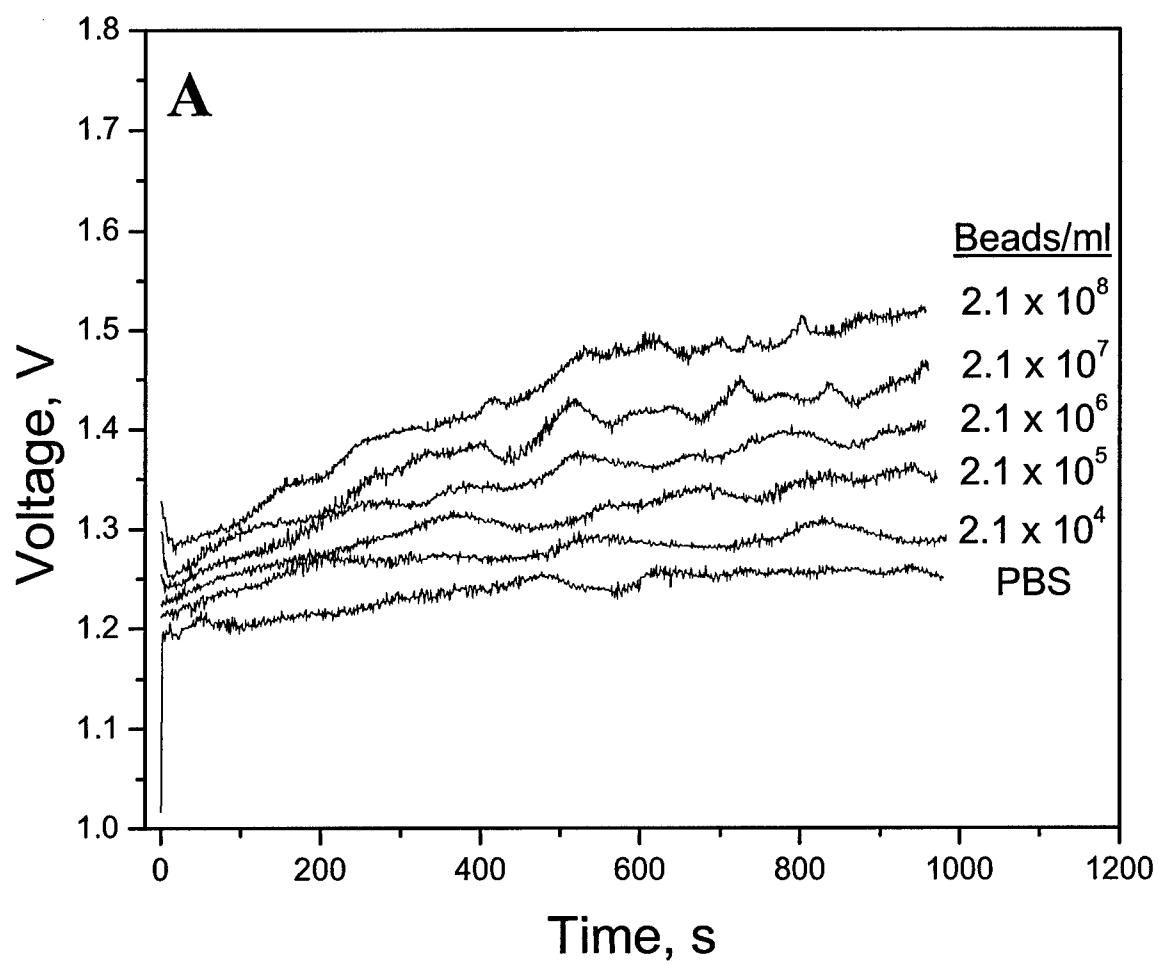
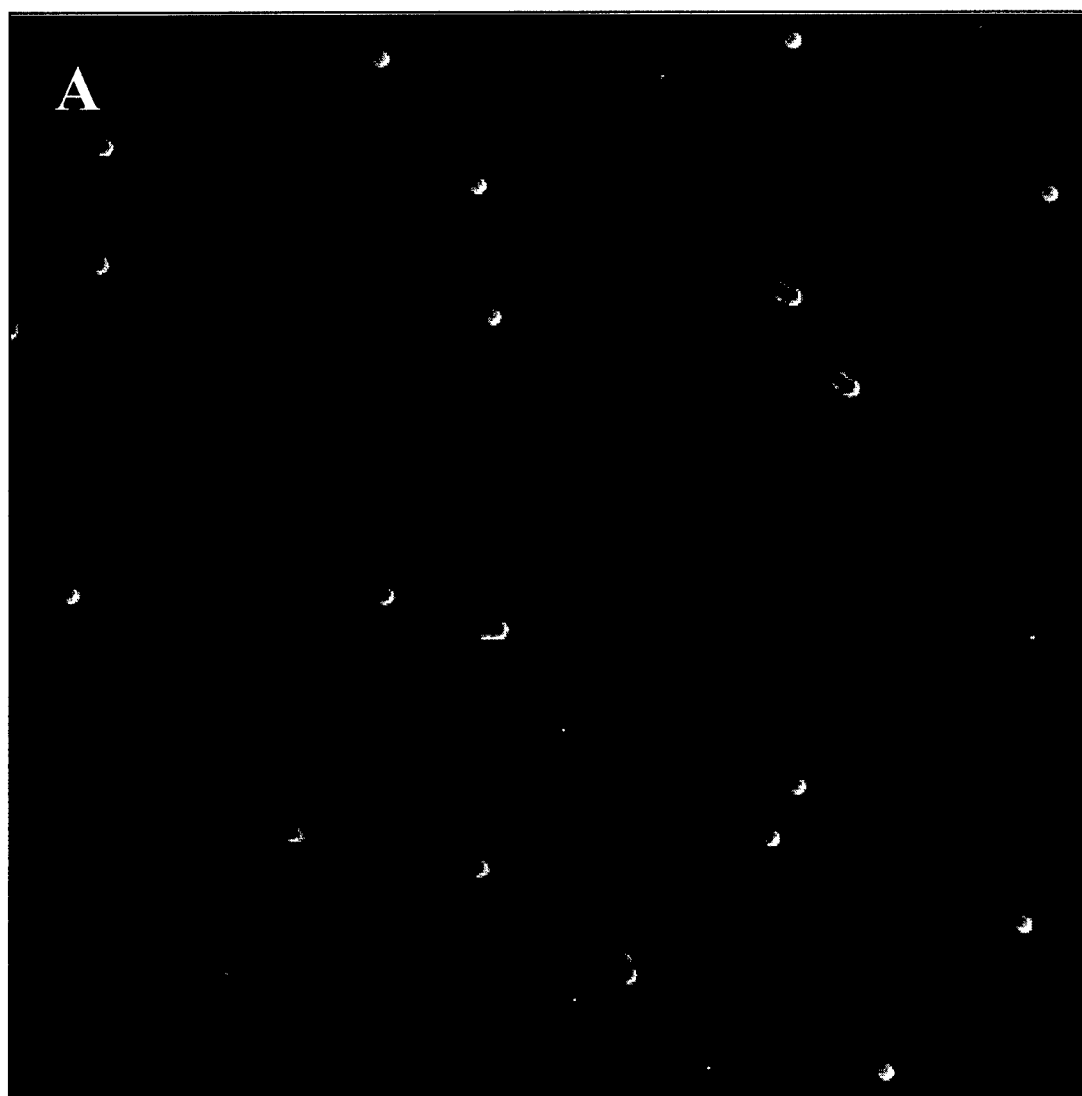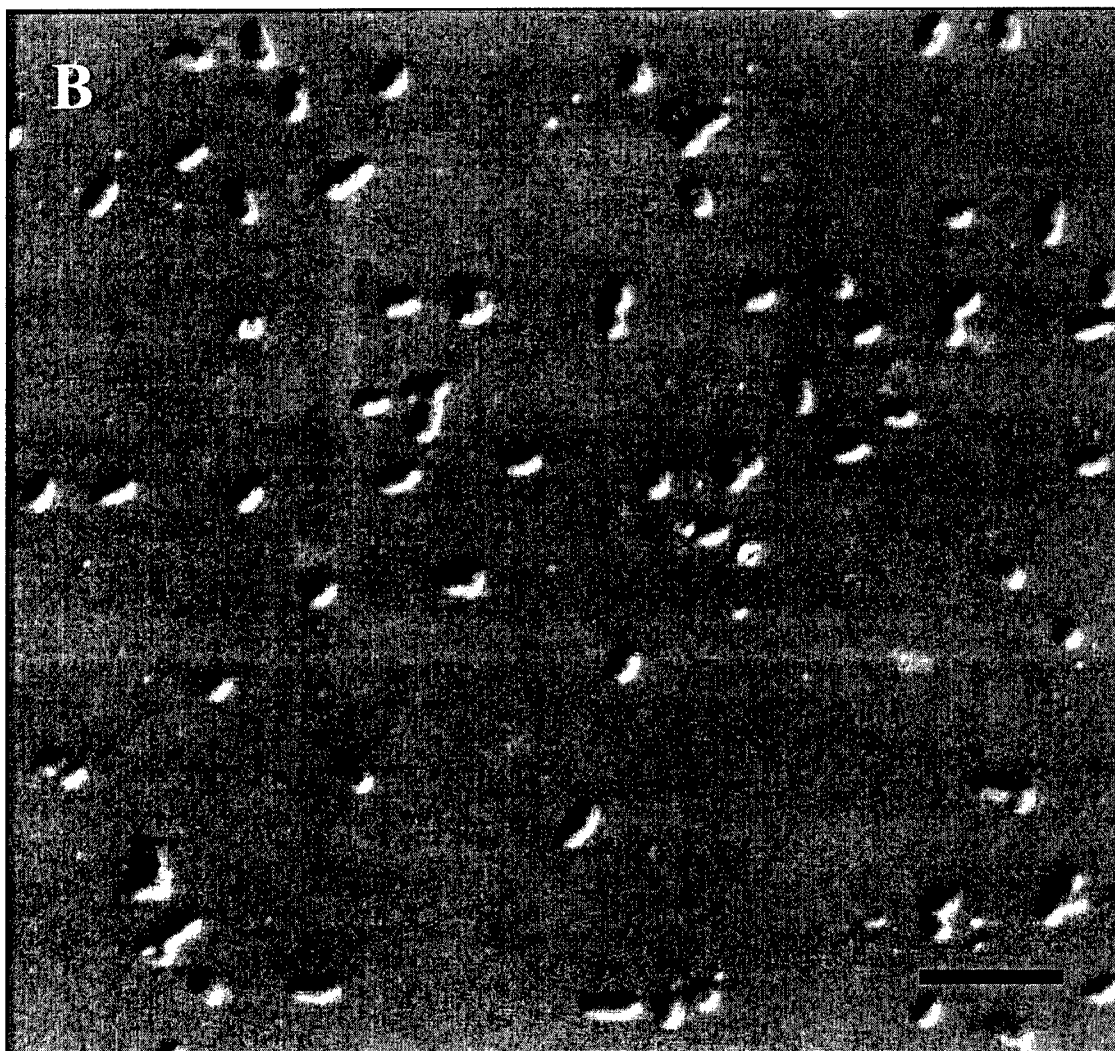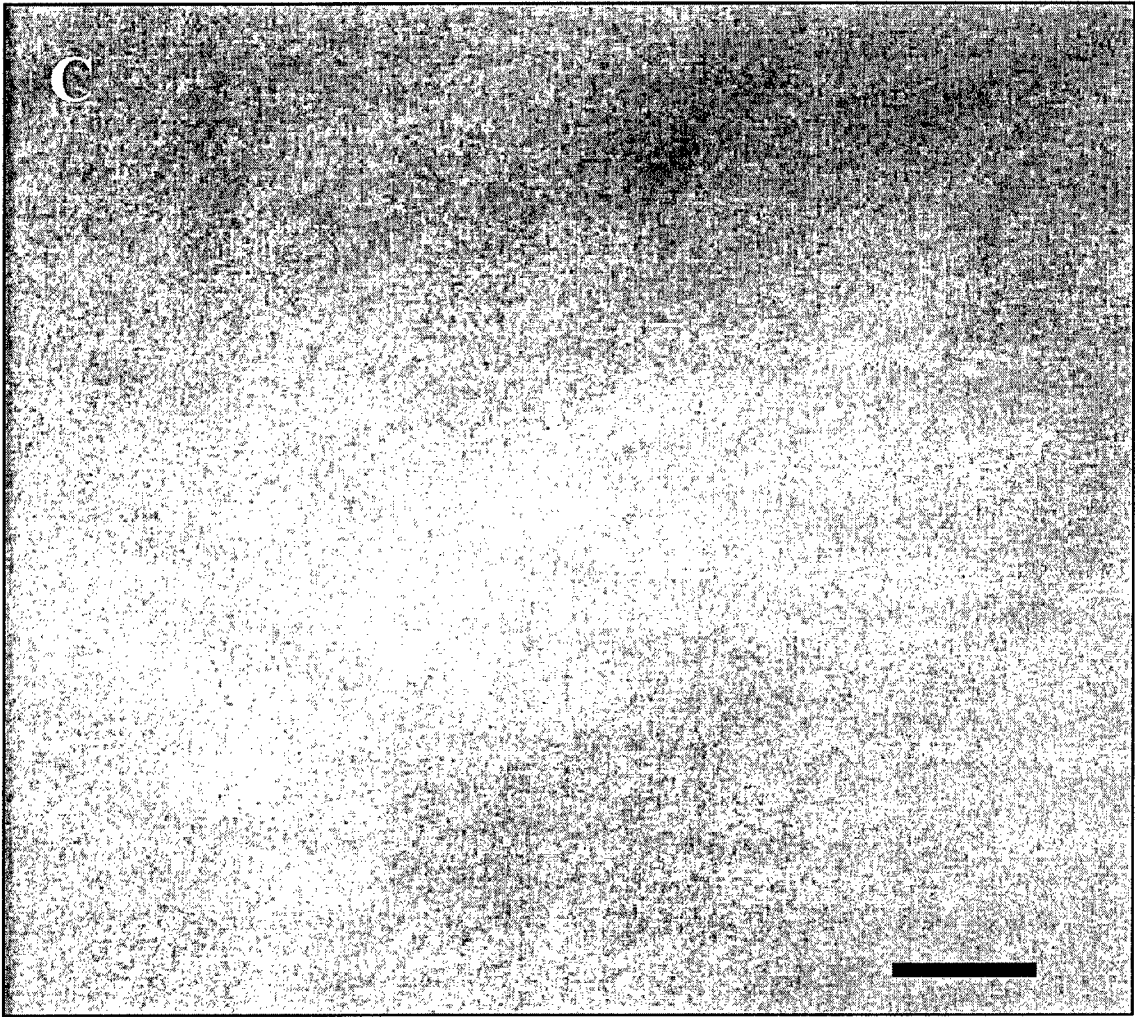Fig. 8B
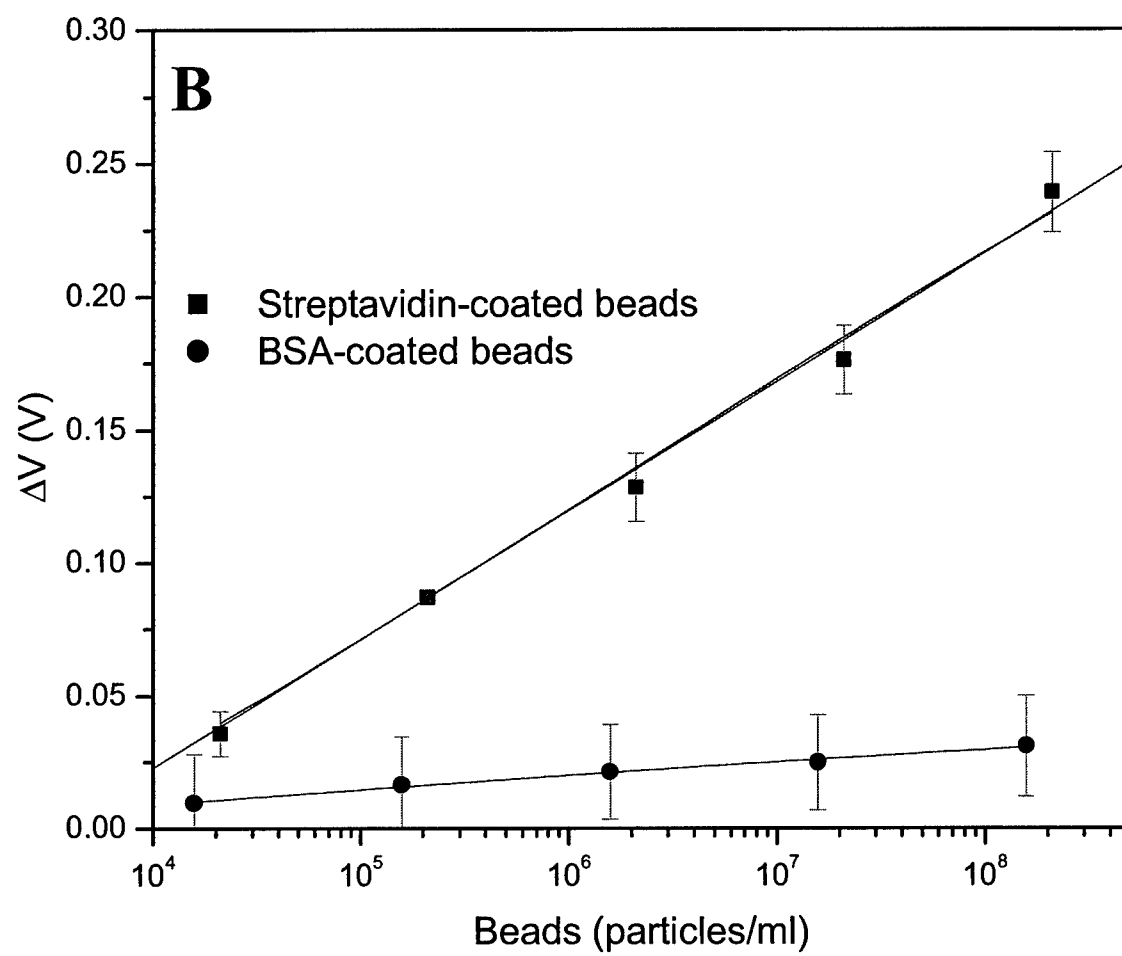
Fig. 9A

Fig. 9B

Fig. 10A

Fig. 10B

Fig. 10C

Figure Legends

Fig. 1. (A) Phage – alkaline phosphatase-labeled streptavidin (APSA) binding measured by direct ELISA. Sigmoidal fit to ELISA data points curve indicates dose-dependent binding (R^2 = 0.997) between 7b1 (test) phage and APSA, while f8-5 (control) phage demonstrated no binding. (B) Competitive ELISA assay measured phage binding to APSA in the presence of streptavidin. APSA concentration was constant; streptavidin concentration varied.

Fig. 2. 0.8% Agarose gel electrophoresis (AGE) of filamentous phage and spheroids. (A) Phage and spheroids treated (right) and non-treated (left) with NaOH. Lanes: 1 and 8, *BstE* II λ DNA ladder. 2 and 7: Filamentous phage not subjected to chloroform treatment. 3 and 6: Filamentous phage subjected to chloroform denaturation (spheroids). Lanes 1–3 were subjected to NaOH treatment prior to staining; lanes 5–8 were not subjected to NaOH treatment. (B) Spheroid induction as a function of chloroform exposure duration (1 or 3 min) and filamentous phage dilution (neat, 3×, 6×). Lanes: M, *BstE* II λ DNA ladder. 1 – 3: 1 min vortex. 4 – 7: 3 min vortex. 1 and 4: neat phage. 2 and 5: 3× dilution. 3 and 6: 6× dilution. 7: solvent-phage interface from prep 1 (lane 1). (C) Spheroid induction as a function of filamentous phage dilution only. All chloroform treatments - 1 min. Lanes: M, *BstE* II λ DNA ladder. 1: neat phage E2 (not subjected to chloroform treatment). 3: 3× dilution. 4: 9× dilution. 5: 27× dilution. 6: 81× dilution. 7: 243× dilution.

Fig. 3. Transmission Electron Microscopy (TEM) (A) 7b1 filamentous phage induced to spheroids (S) by chloroform treatment. Magnification, × 302,500. (B) E2 Filamentous phage induced to spheroids by chloroform treatment. Examples of both I-forms (I) and spheroids (S) are evident following preparation at room temperature. Magnification, × 195,300; bar = 100 nm. (C) Chloroform denatured phage E2 filaments (F), I-forms (I) and spheroids (S) are evident, as is spheroid DNA extrusion (N). Magnification, × 123,800; bar = 100 nm. (D) Spheroids readily adjoining *S. typhimurium* in aqueous solution. I-forms are also apparent. Magnification, × 68,800; bar = 1μm. (E) E2 filaments in bundles, as well as spheroids, are apparent in the interface phase between the solvent (chloroform) and aqueous phase. This photo corresponds to Fig. 2B, lane 7 - bands "A" and "B." No spheroids were noted in the aqueous phase of this sample (Lane 1, Fig. 2B). Magnification, × 30,300; bar = 1μm.

Fig. 4. DNASTAR analysis of pVIII phage coat protein. (A) α-helical regions (Garnier-Robson method). (B) α-helical regions (Chou-Fasman method). (C) Hydrophilicity plot. (D) α-helical amphipathic regions. (E) β-sheet amphipathic regions. (F) Flexible regions. (G) Antigenic index.

Fig. 5. Hypothetical orientation of pVIII coat proteins following rupture of spheroids at the air-liquid (subphase) interface N: pVIII N-terminus. C: pVIII C-terminus. Cylinder represents pVIII hydrophobic domain.

Fig. 6. Hypothetical arrangement of skinned phage as deposited to hydrophilic QCM substrates by Langmuir-Blodgett.

Fig. 7. (A) Π–*A* isotherms of biopolymers as a function of compression at 20 °C. AA: Arachidic acid. 7b1: 7b1 spheroids. E2: E2 spheroids. E2-PC: E2 spheroids reconstituted phospholipid. SAB: *Salmonella* polyvalent-O antibodies. (B) Elasticity of monolayers as a function of surface pressure at 20°C (designations same as 7A).

Fig 8. (A) Voltage responses of streptavidin QCM biosensor to increasing concentrations of streptavidin-coated beads as a function of time. (B) Differential dose-response relation of the mean values (n = 120) of steady-state output sensor voltages as a function of streptavidin– (■) and BSA–coated (●) bead concentrations. Bars are SD. Curves are linear least squares fit to experimental data (streptavidin: $R = -0.997$ slope = 48.6 mV, p <0.05).

Fig. 9. Experimental correlation coefficient as a function of sensitivity for (A) 15 *Salmonella* biosensors prepared with E2 spheroids and (B) 24 biosensors prepared with E2 spheroids in PC by LB method. Correlation coefficients, *r*, were derived from the linear fit to dose-response signals for each tested sensor; sensitivities from the slope of the linear fit.

Fig. 10. Scanning electron microscopy. (A) Streptavidin-coated polystyrene beads (1 μm in diameter) bound to surface of QCM coated with phage coat protein monolayer.

Magnification, × 1000. (B) *S. typhimurium* binding to phage coat proteins deposited to the surface of a QCM by LB. Magnification, × 1000; bar = 10 μm. (C) Control sensor with only phage monolayer deposited. The smooth surface is indicative of a polished resonator. Magnification, × 1000; (black) bar = 10 μm